The effect of temperature on circulating levels of methyl farnesoate and ecdysteroid hormones in female snow crab *Chionoecetes opilio*, from the Bering Sea

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THE EFFECT OF TEMPERATURE ON CIRCULATING LEVELS OF METHYL FARNESOATE AND ECDYSTEROID HORMONES IN FEMALE SNOW CRAB *CHIONOECETES OPILIO*, FROM THE BERING SEA

A

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Abstract

Methyl farnesoate, a reproductive hormone in crustaceans, was detected for the first time in the hemolymph of female *Chionoecetes opilio*. Ovigerous crabs were maintained at -1, 0, 1, and 3° C to represent near bottom temperatures of the Bering Sea, throughout a reproductive cycle to assess changes, and temperature effects, in hormone levels associated with ovarian maturation, hatching and subsequent extrusion of the next clutch. Methyl farnesoate (MF) levels did not change significantly in any treatment throughout the duration of egg incubation. Levels of MF significantly decreased in crabs exposed to -1 and 0° C when initial versus post extrusion levels were compared. Ecdysteroid levels remained low throughout the egg incubation period but increased significantly after extrusion in all treatments except the 0° C treatment. Decrease in temperature significantly prolonged time to extrusion between all temperature groups except 3 and 1° C. Extrusion was delayed in the 0 and -1° C treatments when compared to the 3 and 1° C treatments. The duration of extrusion increased by as much as 77% (between -1 and 0° C) as temperature decreased, suggesting changes in temperature can have a significant effect on the reproductive timing in *C. opilio*.

Table of Contents page

Table of Contents (continued) page

List of Figures page

List of Tables page

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General Introduction

Snow crabs (Family Oregonidae) are a commercially important species of crab harvested in the northwest Atlantic, Sea of Japan, and the Bering Sea (Elner and Benninger, 1992). In 1991 the snow crab fishery in the eastern Bering Sea, one of the most valuable fisheries of the United States, had a peak harvest of 329 million pounds (Figure 1) valued at $165 million (Bowers, 2001). Annual snow crab catch has fluctuated widely (Zheng *et al.*, 2001). Steady decreases in estimated adult biomass since 1991 resulted in the population falling below the minimum biomass threshold and the fishery was declared "over fished" by National Marine Fisheries Service in 1999. The critical state of the Alaskan snow crab *Chionoecetes opilio* (J.C. Fabricius, 1788) fishery in the Bering Sea in the late 1990's punctuated the need and provided the impetus to characterize their biology and reproduction in order to better understand their population fluctuations and thereby improve management strategies. Funds to restore the fishery were subsequently appropriated by the U.S. Congress under a program entitled Bering Sea Snow Crab Restoration Research, and applied to the State of Alaska for cooperative state and federal research (Rugolo *et al.*, 2004).

The population declines and the collapse of some fisheries around the world over time have initiated debate on causation. Over fishing and climate change are suspected causes. Complex factors involved in climate forcing are being studied extensively as trends in climate change are known to perturb fish populations and prey availability (Brodeur and Ware, 1992; Anderson *et al.*, 1997; Sugimoto and Tadokoro, 1997). Understanding population fluctuations is critical in order to manage a sustainable fishery.

Research on the biology and life history of the species being exploited is necessary to understand the parameters that govern recruitment to the fishery and ascertain what type of fishing pressure the resource can withstand (Kruse, 1996; Orensanz *et al.*, 1998; Zheng and Kruse, 2000). In addition, increasing the information available concerning the ecologically important region of the Bering Sea may well prove useful as resource exploration and development activities increase there (Jewett, 1981).

General Distribution

Chionoecetes opilio is a cold water species (Foyle and O'Dor, 1989) inhabiting discreet circumboreal areas of the globe (Hart, 1982). *Chionoecetes opilio* inhabit the Japan Sea east of the Korean Peninsula, the Sea of Okhotsk, Bering Sea, and the northwest Atlantic Ocean, ranging between 60 to 400 m in depth (Squires, 1969; Elner and Benninger, 1995). In the Bering Sea their benthic habitat is reportedly between -1 and 3° C, which can vary with fluctuating sea ice coverage (Luchin *et al.*, 1999). They inhabit muddy or sandy environments on the continental shelf in the Bering Sea at depths of 50 to 150 m (Zheng *et al.*, 2001).

During the past three decades, the distribution of *C. opilio* in the Bering Sea has changed and contracted from south to north corresponding to near bottom temperature (NBT) increases, due to climate change (Orensanz *et al.*, 2004). These persistent climate changes reverse the previous climate pattern (Trenberth and Hurrell, 1995), causing fluctuations in fish prey availability due to fluctuating ocean primary productivity (Brodeur and Ware, 1992; Klyashtorin, 1997; Anderson and Piatt, 1999). Consequent

changes in NBT together with cascade effects throughout trophic levels in the water column are thought to have caused *C. opilio* to shift northward in the Bering Sea (Brodeur and Ware, 1992).

General Reproduction

Recruitment variability of Atlantic *C. opilio* in the Gulf of St. Lawrence, on the eastern Canadian coast is related to lengthened clutch maturation at colder seawater temperatures (Mallet *et al.*, 1993; Sainte-Marie, 1993). The duration of clutch incubation for female *C. opilio* is slower in the colder, shallower (-1 to 1° C at 40 -100 m) waters of the Gulf of St. Lawrence where egg incubation is between 24 to 27 months (Lovrich *et al.*, 1995; Conan *et al.*, 1996; Sainte-Marie and Gilbert, 1998). In the warmer, deeper (1.8 to 3.8° C at 100-300 m) waters the clutches mature between 12 -13.5 months (Mallet *et al.*, 1993; Moriyasu and Lanteigne, 1998; Sainte-Marie and Gilbert, 1998; Comeau *et al.*, 1999). Bering Sea *C.opilio* experience temperatures averaging between -1 to 3° C (Luchin *et al.*, 1999; Zheng *et al.*, 2001), which could prolong the incubation period (Sainte-Marie and Hazel, 1992; Lovrich *et al.*, 1995) causing a biennial reproduction cycle as seen in the Atlantic populations (Moriyasu and Lanteigne, 1998), ultimately resulting in weak recruitment years (Elner and Benninger, 1992).

A large *C.opilio* fishery exists in the Gulf of St. Lawrence (GSL) on the Canadian Atlantic coast. Females from the GSL have two maturation states (Elner and Benninger, 1992) defined as primiparous, a female with her first clutch of eggs, or multiparous, a female that has produced more than one clutch. Differences between these female

maturation states temporally influences reproductive events such as mating, duration of clutch incubation, and fecundity (Elner and Benninger, 1992; Sainte-Marie and Hazel, 1992; Sainte-Marie, 1993; Comeau *et al.*, 1999). Primiparous females invest energy in growth as they increase in mass after their terminal molt. Primiparous females produce smaller clutches than multiparous females that no longer expend energy on growth (Elner and Benninger, 1995). In the GSL, mature males (usually individuals that have yet to undergo the terminal molt) mate with primiparous females early in the year (February) and terminally molted males mate in the spring to early summer with multiparous females (Conan and Comeau, 1986; Moriyasu and Lanteigne, 1998).

Sperm, deposited by the male into the female during copulation, is either used to fertilize the imminent clutch of eggs or stored for future clutch fertilization (Benninger *et al.*, 1993). Multiparous females are capable of fertilizing clutches by utilizing sperm stored in the paired spermatheca (Paul, 1984), which could offset sperm limitations in years of low male abundance or periods of increased competition due to high female abundance. Egg extrusion generally occurs shortly after copulation (Ennis *et al.*, 1988). During egg extrusion, the oocytes move from the ovaries to the abdominal pleopods (Elner and Benninger, 1992) and in *C. opilio*, egg extrusion occurs within a matter of days after egg hatching (Webb *et al.*, 2006). Eggs are fertilized as they pass over the spermathecal ducts during the course of extrusion (Elner and Benninger, 1992). Generally, female snow crabs undergo ovarian maturation while brooding a clutch (Benninger *et al.*, 1993).

Hormonal Control of Growth and Reproduction

Crustacean reproduction is regulated by hormones which are compounds synthesized by endocrine glands and secreted into the bloodstream where they circulate at low concentrations. Circulating hormones bind to specific receptor sites located on the target cell, generating a chemical message that causes a change in the long-term biochemical and/or physiological activity of that cell. Crustacean growth (molting) and reproduction are interrelated, complex, hormonally controlled processes (Skinner, 1985; Meusy and Payen, 1988; Chang *et al.*, 1993; Laufer *et al.*, 1996; Reddy and Ramamurthi, 1999).

The eyestalks of decapod crustaceans contain the x-organ-sinus gland (XO-SG) complex, which is a neuroendocrine gland. Neurohormones produced within the x-organ are stored and released by the sinus gland (Carlisle and Pasano, 1953). Through the release of inhibitory neuropeptides, the XO-SG regulates diverse physiological processes such as color change, and metabolism as well as energetically opposing processes like growth and reproduction (Pasano, 1953; Chang *et al.*, 1993; Reddy and Ramamurthi, 1999). One such neuropeptide, molt-inhibiting hormone (MIH), negatively regulates the endocrine function of the y-organ which synthesizes and secretes ecdysteroids associated with growth (Chang *et al.*, 1991). When MIH is removed by extirpation of the eyestalks, the molt cycle is accelerated (Snyder and Chang, 1986; Chang *et al.*, 1993) and the circulating levels of molt promoting hormones increase (Tamone *et al.*, 2005).

A less studied role of the XO-SG in the regulation of reproduction, involves the release of mandibular organ inhibiting hormone, a neurohormone that inhibits the activity

of the mandibular organ (MO; Borst *et al.*, 2001). The paired MO is located at the base of the maxillary tendons (Le Roux, 1968). The MO synthesizes methyl farnesoate (MF), a gonadotropic hormone in male and female crustaceans (Laufer *et al.*, 1987b). The MO is homologous to the corpus allatum endocrine gland found in insects (Byard *et al.*, 1975) which produces a homologous MF sesquiterpenoid hormone; juvenile hormone III . Gametogenesis and metamorphosis in insects are known to be regulated by derivatives of juvenile hormone (Gilbert *et al.*, 2000).

Methyl farnesoate was the first hormone identified by reverse endocrinology. Modern molecular techniques allow researchers to identify endocrine function of compounds in reverse order from the classical approach. The classical method which typically proceeds with extirpation of the tissue, histologic inspection of the tissue for endocrine structure and reversal of the change to the organism by reimplantation of the tissue or injection of an extract thereof (Reddy and Ramamurthi, 1999). Methyl farnesoate was confirmed as a secretory product of the MO in *Libinia emarginata* (Laufer *et al.*, 1986) and was characterized from hemolymph via gas chromatography/mass spectroscopy (Laufer *et al.*, 1987b).

Methyl farnesoate has been shown to positively regulate growth by acting on the y-organ to stimulate ecdysteroid secretion (Hinsch, 1981b; Tamone and Chang, 1993). Methyl farnesoate is also known for its regulatory role in reproductive processes in crustaceans (Borst *et al.*, 1987; LeBlanc *et al.*, 1999). Implants of MO extracts from males into juvenile females stimulated ovarian development in *L. emarginata* and increased morphological changes in the MO as well as initiating ovarian maturation

(Laufer *et al.*, 1986; Laufer *et al.*, 1987b). Methyl farnesoate fed to shrimp increased production of offspring (Laufer *et al.*, 1986). The increased rate of MF secretion by the mandibular organ in *Libinia emarginata* correlates with progression of ovarian growth (Laufer *et al.*, 1987b). The swamp crayfish *Procambarus clarkii* exhibits increased oocytic growth as a result of MF injections at the adult stage (Rodriguez *et al.*, 2002). Injections of MF into the freshwater crab *Oziotelphusa senex senex* induced vitellogenesis (Reddy *et al.*, 2004). Methyl farnesoate initiates sexual reproduction in *Daphnia*, which are parthenogenic under normal environmental conditions and revert to sexual regeneration only during times of environmental stress (Rider *et al.*, 2006).

Vitellogenesis

Oocytes are produced during ovarian maturation from the germinative layer of the ovary through, oogenesis. During oogenesis the developing eggs begin to sequester yolk proteins or vitellin (Vn). Vitellin is produced in the ovary from circulating vitellogenin (Vg), the extraovarian precursor molecule (Meusy, 1980). During the latter stages of oogenesis, oocytes take up Vn as their primary nutritive source (Meusy and Payen, 1988).

Vitellogenic *L. emarginata* exhibit increased MF levels which correlate to increased gonadal somatic index (GSI). After oviposition (egg extrusion), MF levels decrease (Laufer *et al.*, 1996), suggesting a regulatory role for MF on vitellogenesis. Characterization of circulating MF levels during vitellogenesis and oviposition may provide an indication of the hormonal regulation of ovarian maturation.

Ecdysteroids and Reproduction

Ecdysteroid secretion by the y-organ is stimulated by MF (Tamone and Chang, 1993), which suggests either a role for MF in growth, or a role for ecdysteroids in crustacean reproduction, or perhaps both. In male *L. emarginata* approaching terminal and morphometric molt, both MF and ecdysteroid levels are elevated, while levels in males that have undergone their terminal molt have low ecdysteroid levels (Laufer *et al.*, 2002). Low levels of circulating ecdysteroids in terminally molted males is attributed to the degeneration of the ecdysteroid producing y-organ in species that undergo a terminal molt (Cormier *et al.*, 1992; Gunamalai *et al.*, 2004).

Sequestration of ecdysteroids in the ovary of *Emerita asiatica* during ovarian maturation increases with a corresponding decrease in circulating ecdysteroids (Gunamalai *et al.*, 2003). Increased ecdysteroids in the ovary of mole crabs correspond with timing of embryo hatch (Gunamalai *et al.*, 2004). We analyzed ecdysteroid levels as well as levels of MF to assess the relationship, if any, between these hormones and ontogeny of ovarian maturation.

Hypotheses

Of the various factors affecting endocrine activity such as nutrition, photoperiod, and stress, we focus here on temperature effects in order to indirectly assess ovarian maturation of female *Chionoecetes opilio* from the Bering Sea by characterizing circulating MF and ecdysteroid levels, from hemolymph samples. Non-lethal sampling

methods allow blood samples to be acquired in the field and subsequently analyzed in the laboratory.

Ho: Ovarian maturation or "vitellogenesis" in *Chionoecetes opilio* as measured indirectly by methyl farnesoate levels does not change with temperature.

Ha: Ovarian maturation or "vitellogenesis" in *Chionoecetes opilio* as measured indirectly by methyl farnesoate levels changes at different temperatures.

Ho: Ecdysteroid levels have no correlation to ovarian maturation in *Chionoecetes opilio*.

Ha: Ecdysteroid levels correlate with ovarian maturation in *Chionoecetes opilio*.

Chapter 1

* The effect of temperature on circulating levels of methyl farnesoate and ecdysteroid hormones in female snow crab *Chionoecetes opilio,* from the Bering Sea

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INTRODUCTION

In 1999 the snow crab, *Chionoecetes opilio* (J.C. Fabricius, 1788) population in the eastern Bering Sea, one of the most valuable fisheries in the United States, declined to a level below the minimum threshold to sustain a fishery and was declared "overfished" (Rugolo *et al.*, 2004). Snow crab recruitment has fluctuated widely over the last few decades, while information about their reproductive biology and fecundity has been sparse (Kruse, 1996). Recruitment patterns over the last 27 years have been periodic for crabs in the eastern Bering Sea (EBS) with approximately eight years between strong recruitment periods (Zheng *et al.*, 2001). The Fishery Management Plan of the North Pacific Fisheries Management Council (NPFMC) establishes a cooperative management system for *C. opilio* that defers certain management controls to the state of Alaska Department of Fish and Game (ADF&G). The National Marine Fisheries Survey (NMFS) conducts the annual survey and supplies all necessary management data to the NPFMC. Once the data has been analyzed the guideline harvest limits are set and subsequently ADF&G manages the fishery accordingly. Harvest levels, from the last two decades, indirectly reflect the oscillating nature of adult snow crab populations in the EBS (Figure 1).

Chionoecetes opilio are cold adapted and confined to specific thermal limits (Foyle and O'Dor, 1989). Mature females tend to occur in an area termed the middle domain of the EBS in close relation to the regions isotherm, defined by the 2° C near bottom temperature (NBT) cold pool (Luchin *et al.*, 1999; Orensanz *et al.*, 2004). In recent years a northward shift in the range of *C. opilio* in the Bering Sea has been

attributed to increasing water temperatures in the south (Anderson and Piatt, 1999; Zheng *et al.*, 2001) and northward shifts in the cold pool (Orensanz *et al.*, 2004).

Reproductive processes in *C. opilio* such as ovarian maturation are directly related to temperature (Lovrich *et al.*, 1995; Conan *et al.*, 1996; Sainte-Marie and Gilbert, 1998). Female *C. opilio* at colder temperatures have a longer gestation period than those at warmer temperatures (Sainte-Marie and Hazel, 1992; Rugolo *et al.*, 2004; Webb *et al.*, 2006), indicating this effect of temperature on the physiological regulation of reproductive processes. Environmental cues may alter physiological processes by altering the rates of metabolic processes. Another way environmental cues affect the physiology of living organisms is through a perturbation of hormones responsible for regulating physiological processes.

Endocrine Regulation

Environmental cues such as photoperiod and temperature regulate the nervous system in living organisms eliciting a physiological response appropriate to the environment. The physiological response may increase the likelihood that the organism will adapt to changes in the environment to promote survival and fitness, for instance, timing reproduction to correspond to favorable environmental conditions for the offspring. The nervous system communicates to the endocrine system through the secretion of neurohormones into the circulation system. The endocrine system can respond to the signal by producing hormones and releasing them into the circulation The regulation of crustacean physiology including growth and reproduction has been

thoroughly reviewed (Skinner, 1985; Meusy and Payen, 1988; Chang *et al.*, 1993; Laufer *et al.*, 1996; Reddy and Ramamurthi, 1999). The primary neurohormones are secreted from the x-organ-sinus gland complex (XO-SG) located within the crustacean eyestalk. Neurohormones produced within the XO are stored and released by the SG (Carlisle and Pasano, 1953). The XO-SG regulates diverse physiological processes such as color change and metabolism, as well as energetically opposing processes like growth and reproduction (Pasano, 1953; Chang *et al.*, 1993; Reddy and Ramamurthi, 1999) and rhythmic functions such as locomotion (Arechiga *et al.*, 1985), through the release of inhibitory neuropeptides that act specifically on endocrine glands to regulate the secretion of specific hormones.

Methyl Farnesoate

Methyl farnesoate (MF) is a reproductive hormone, specifically a gonadotropic sesquiterpenoid, in male and female crustaceans (Laufer *et al.*, 1987b). Methyl farnesoate is a hydrophobic hormone that requires specific binding proteins in order to circulate in the hemolymph. The binding proteins for MF have been identified and characterized in *Cancer magister* (Tamone *et al.*, 1997). Methyl farnesoate is secreted from paired mandibular organs (MO) which are attached to the abductor muscles of the mandibles in crustaceans and were first described by Le Roux (Le Roux, 1968). MF secretion from the MO is regulated by the XO-SG, via the neurohormone, mandibular organ inhibiting hormone (MOIH; Borst *et al.*, 2001). Results of eyestalk ablation experiments in the lobster *Homarus americanus* show that removal of this gland results in increases of

circulating MF, and reimplantation of eyestalk extracts decreases circulating MF (Borst *et al.*, 2001).

Much of what is known about the MO and the role and mechanism of action of MF in crustacean physiology derives from deductive research based on homology with the insect endocrine gland, corpus allatum and its product JH III (Laufer *et al.*, 1987a). Reproductive and developmental processes (gametogenesis, ontogeny, and development) in insects are regulated by derivatives of juvenile hormone (Wilder and Aida, 1995; Gilbert *et al.*, 2000). Methyl farnesoate is the unepoxidated form of JHIII. Methyl farnesoate is a secretory product of the MO in *Libinia emarginata* (Laufer *et al.*, 1987a) and it was first characterized from crab hemolymph using gas chromatography/mass spectroscopy (Laufer *et al.*, 1987b).

The physiological role for MF in crustacean physiology is most often correlated with reproduction (Borst *et al.*, 1987; Laufer *et al.*, 1992; Subramoniam and Keller, 1993; Wilder and Aida, 1995; Reddy and Ramamurthi, 1998; Reddy and Ramamurthi, 1999; Laufer and Biggers, 2001; Reddy *et al.*, 2004), but it also has a documented role in growth (Tamone and Chang., 1993; Laufer *et al.*, 1997) and stress (Lovett *et al.*, 2001). The rate of MF secretion by the MO in *L. emarginata* increases with the progression of ovarian growth, and varies considerably between individuals that differ in reproductive maturity (Laufer *et al.*, 1987b).

Physical changes in the MO are correlated to progression of ovarian maturation (Hinsch, 1981a). Mandibular organs increase in weight with increasing gonadal somatic index (GSI) a ratio of gonadal tissue weight to total tissue weight. This increase in

weight is positively correlated with increased MF content of the MO (Nagaraju *et al.*, 2004). Implants of MO extracts from male *L. emarginata* into juvenile females increased morphological changes in the MO as well as initiating ovarian maturation (Hinsch, 1980; Laufer *et al.*, 1987b; Laufer *et al.*, 1987a). Methyl farnesoate fed to shrimp increased reproduction (Laufer *et al.*, 1987a). Injections of MF into swamp crayfish, *Procambarus clarkii* increased oocytic growth (Rodriguez *et al.*, 2002), and in the freshwater crab *Oziotelphusa senex senex* induced vitellogenesis (Reddy *et al.*, 2004). In a parthenogenic species of *Daphnia,* MF induced sexual reproduction (Rider *et al.*, 2006).

Ovarian maturation involves the production of oocytes (oogenesis) and their subsequent uptake of yolk protein, vitellin (Vn), in a process called vitellogenesis. During the latter stages of oogenesis, maturing oocytes begin to sequester Vn, the primary nutrient source for the future embryo, whereby circulating vitellogenin (Vg) is taken up by the ovaries and converted into Vn by the addition of polysaccharides and lipids prior to uptake. Vitellin is synthesized from the precursor molecule, Vg (Meusy, 1980). Within the Brachyura, Vg is thought to be synthesized by ovarian and hepatopancreas tissue (Meusy and Payen, 1988). In *C.opilio*, Vg circulates in the hemolymph and it is unclear if it is derived from the hepatopancreas or the ovary (Tamone, unpublished data).

The genes that transcribe Vg in insects are known to be stimulated by JH III, the MF homologue (Engelmann, 2002). In the spider crab *L. emarginata* elevated MF levels in vitellogenic females correlate with increased GSI (Laufer *et al.*, 1996). After

oviposition, circulating MF decreases, supporting a regulatory role for MF in vitellogenesis.

MF has been successfully quantified in *L. emarginata*, *Homarus americanus,* and *Carcinus maenas* by normal-phase high performance liquid chromatography, with sensitivity in detection levels reportedly below 250 pg/ml (Borst and Tsukimura, 1991). Methyl farnesoate has also been successfully quantified by HPLC in *Nephrops norvegicus* (Rotllant *et al.*, 2001). Characterizing MF in female snow crabs may help determine the normal timing of the reproductive cycle in snow crabs. Further usefulness may include the ability to monitor reproductive timing in snow crab of a certain geographical region based on regional near bottom temperatures.

Ecdysteroids and Growth

Crab growth occurs through the process of ecdysis that requires formation of a new cuticle or exoskeleton and terminates in shedding of the old exoskeleton. In both male and female snow crabs, growth occurs until a terminal molt, after which the crab no longer molts or increases in size, although crabs will continue to gain mass to fill their terminal exoskeleton (Tamone *et al.*, 2005). Molting is regulated by stimulatory molting hormones (ecdysteroids) and inhibitory molting hormones (Chang *et al.*, 1993). Molting in crustaceans is hormonally controlled by the steroid 20-hydroxyecdysone (20-HE) which is synthesized from ecdysone secreted by the y-organ. Molt-inhibiting hormone (MIH) negatively regulates the endocrine function of the y-organ (Chang *et al.*, 1991, Chang *et al.*, 1993; Chang *et al.*, 1995).

Methyl farnesoate increases ecdysteroid secretion from the y-organ in vitro (Tamone and Chang, 1993). Both ecdysteroids and methyl farnesoate levels increase in male *L. emarginata* prior to ecdysis (Laufer *et al.*, 2002). Ecdysteroids in male *L. emarginata* that have undergone their terminal molt, circulate at relatively low concentrations (Laufer *et al.*, 2002); this is considered to be a result of the degeneration of the y-organ after terminal molt (Cormier *et al.*, 1992). Terminally molted male *C. opilio* also exhibit low levels of ecdysteroids which cannot be induced through eyestalk ablation (Tamone *et al.*, 2005). The MOs enlarge during the molt cycle (Hinsch, 1981a), suggesting a regulatory role for MF in crustacean molting.

Ecdysteroids and Reproduction

The role of ecdysteroids in crustacean reproduction is not clear. Ecdysteroids released into the environment during the molt process are thought to act as sexual attractants (Kitteredge and Takahashi, 1971). Attractant pheromones have been studied in male snow crabs (Bouchard *et al.*, 1996); males are attracted to olfactory cues from pubescent females as well as multiparous females stripped of eggs. Circulating ecdysteroids are conjugated to polar compounds by the hepatopancreas prior to excretion and these polar compounds are bioactive (Snyder *et al.*, 1992). Ecdysteroids are found in the eggs and ovaries of most crustacean species, and their levels have been observed to peak in correlation with specific embryonic stages and hatching (Wilder and Aida, 1995).

Increased ecdysteroid levels are found in the eggs of ovigerous female crabs, *Cancer productus* and *Cancer anthonyi* during egg incubation (Okazaki *et al.*, 1988).

Ecdysteroids are sequestered in the testes of male shore crabs *Pachygrapsus cassipes*, supporting a role for ecdysteroids in reproduction perhaps by regulation of spermatogenesis (Chang *et al.*, 1976) .

Circulating ecdysteroids and methyl farnesoate levels were measured over the course of a reproductive cycle to better understand how temperature may affect the role that these hormones play in snow crab ovarian maturation, hatching and egg extrusion.

Materials and Methods

Female snow crabs were collected from the Bering Sea, during the Alaska Department of Fish and Game survey using the chartered vessel, FV BILIKEN, from a region south of St. Mathews Island (58° N, 173° W) at a depth of 117 m with bottom temperatures between 1 and 3° C, and brought into Dutch Harbor in tanks with circulating seawater on August 22, 2001. Crabs were transported to the Juneau Center School of Fisheries and Ocean Sciences (JCSFOS) by air in ice chests kept moist with burlap bags soaked in sea water, and cooled with blue ice. The crabs were placed into 700 L flow-through tanks at ambient sea water temperature for approximately two months until the experimental tanks were equipped with chillers. Weight, carapace width and chela height, abdomen width, shell condition, egg condition, egg color, clutch fullness, and leg status (missing or damaged) was recorded for each crab.

Crabs were segregated into tanks using a stratified random design in order to represent each size class in each tank. Tanks were maintained with flow-through seawater chilled to the experimental temperatures of -1, 0, 1 and 3° C using chillers

(Frigid Units™ Inc. Model D1-33). Temperatures were gradually adjusted to the experimental temperatures. Temperatures were continually monitored using Stowaway Tidbit™ temperature loggers (Onset Computer Inc.). The crabs were fed herring once a week ad libitum; excess food was removed after 24 hours.

The reproductive status of female crabs (primiparous vs. multiparous) was determined by evaluating shell condition. New shelled crabs were considered primiparous and were excluded from this experiment.

Hemolymph Sampling, Hemolymph Sample Preparation and Hormone Analysis

Hemolymph samples (~1.0 ml) were withdrawn monthly (from the base of the 4th walking leg between the carapace and coxa) with a 26-gauge needle and 1.0 ml syringe. Hemolymph samples were stored in cryovials at -20° C prior to hormone and vitellogenin analysis.

Methyl farnesoate and ecdysteroids were extracted from hemolymph samples in a two step procedure. Hemolymph samples were thawed on ice and a subsample (50 µl) removed for future vitellogenin assay. The remaining hemolymph was volumetrically measured by pipette and added to a volume of methanol equal to 75% (3 parts MeOH, 1 part hemolymph), in a borosilicate test-tube (Burdick and Jackson, HPLC grade). The tubes were briefly vortexed. A volume of 200µl of hexane (Burdick and Johnson, HPLC grade) was added to each test-tube and briefly vortexed followed by a 10 minute centrifugation (IEC HN SII at 4° C, 1000g, rotor 958 with 18x15ml spindle). The hexane layer was removed by capillary pipet from the tri-phasic solution into a 600µl full

recovery HPLC sample vial (Waters Corp). The methanolic extract was again extracted with 200µl of hexane and the hexane fractions combined. The methanolic fraction was retained in 1.5 ml eppendorf tubes and stored at -20° C for ecdysteroid analysis.

High Performance Liquid Chromatography

To each sample 20 ng of exogenous cis-trans MF in 100 µl hexane was added to each sample as an internal standard. The concentration of this standard was measured with a spectrophotometer (Shimadzu, deuterium lamp, quartz cuvette) in 1.0 ml MeOH. The concentration of the internal standard was calculated using the extinction coefficient of methyl farnesoate 14,800 moles/liter at 217 λ, according to Beer's Law. The hexane fraction containing exogenous and endogenous MF was evaporated (Sorvall Speed Vac) until dryness. The lyophylized analytes were subsequently re-suspended in a known volume of hexane (either 110µl or 220µl) to standardize the sample volume for HPLC analysis. Samples were loaded onto the auto sampler with a hexane blank followed by a mixed cis-trans and all-trans MF standard (ct/at MF). This mixed standard was repeatedly injected throughout the duration of the run, interspersed between samples, in order to detect variation in retention time.

Methyl farnesoate concentrations were analyzed using normal phase high-performance liquid chromatography (np-HPLC; Waters binary HPLC pump 1525, Waters 717 Plus Autosampler, Waters 2487 dual λ absorbance detector) as described previously (Borst and Tsukimura, 1991). Briefly, hemolymph extracts were separated using a silica column (Alltech Econosil Si 5µl 250 mm x 4.6 mm) in combination with a compatible

anterior guard column (4.0 x 20 mm 120 angstrom, YMC), the mobile phase (sparged with medical grade helium) consisted of hexane with 1% diethyl-ether, using an isocratic flow rate of 2.5 ml/min.

Methyl farnesoate peaks were detected at 217 λ. Cis-trans MF, the internal standard, elutes approximately 1 minute before the all-trans MF, thereby giving a locale on the chromatogram for the unknown peak. Quantification of MF was achieved by first calculating the distance between the ct/at MF peaks of all the standards interspersed throughout a particular run. The retention time differences were critical for selecting the proper peaks on a chromatogram for an unknown sample. Once the relevant peaks were identified integration of the area under each peak was performed (Breeze version 3.2, Waters Corp.) The area under the curve of the internal cis-trans standard of known concentration was related to the area under the all-trans MF peak, the crabs endogenous MF. Correction for loss due to extraction was also made.

Enzyme Linked Immunosorbent Assay (ELISA)

Circulating ecdysteroids in the hemolymph were quantified using a competitive ELISA (Kingan, 1989). The methanol fraction, obtained in methods described above, containing ecdysteroid hormones was evaporated to dryness (Sorvall Speed Vac) and subsequently re-suspended in 500 µl buffer (AB: 25mM sodium phosphate, 150 mM sodium chloride, 1mM EDTA, pH 7.5) containing 0.1% bovine serum albumin (BSA).

Micro titer plates (Costar 96-well plates) were coated with 90µl/well of secondary anti-body, goat anti rabbit IgG (Immuno Jackson Laboratory), in PBS at [0.5µg/90µl]

overnight at room temperature. All wells were blocked with AB/BSA containing 0.002% sodium azide for one hour or overnight and used within two weeks.

A standard curve was generated using 20-hydroxyecdysone (20-HE; Sigma Chemical Company), and diluted with AB/BSA to concentrations that ranged between 1.0 and 500 fm/50µl. Standards and samples were loaded onto the plates (50µl/well). Ecdysone, conjugated to horseradish peroxidase (20 E HRP; obtained from Dr. Tim Kingan, U.C. Riverside) at a dilution of 1:4000 (50 µl) was added to each well. Anti-ecdysone, the primary antiserum (obtained from Dr. Tim Kingan, U.C. Riverside) was diluted 1:100,000, and added to each sample (50µl/well) with the exception of the non-specific binding wells. The plates were allowed to incubate at 4° C overnight. Plates were washed three times with PBS containing 0.05% Tween-20 (Bio Rad 1575 Immunowash), followed by a 1:1 solution of room temperature hydrogen peroxide and tetramethylbenzidine (TMB substrate kit, Kirkegaard and Perry Laboratories). The plates were then stirred by shaking on an orbital shaker for exactly 15 minutes before the reaction was stopped using 1M phosphoric acid (90µl/well). Absorbance was determined at 450 nm, using a spectrophotometer (BioRad micro-plate reader 680, Richmond, CA). Ecdysteroid concentrations were measured by comparing the mean absorbance of the duplicate samples to the standard curve. Standard curves were generated by plotting standard concentrations against the measured absorbance and fitted using a Rodbard regression line (BioRad micro-plate manager version 5.2).

Data Analysis

Circulating methyl farnesoate and ecdysteroids levels in snow crabs prior to and after egg extrusion were compared within temperature treatments by paired t-Tests with α at 0.05, for a 95% confidence interval (Microsoft Office Excel 2003). Extrusion data were analyzed using a one way ANOVA followed by a post hoc Tukey's Studentized Range test, with α at 0.05 (SAS v 8.1).

Results

The survival of crabs in each temperature treatment is shown in Table 1. Survival of crabs sampled for hemolymph in the -1° C treatment was 70%, in the 0° C treatment survival was 90%, in the 1° C treatment survival was 70%, and in the 3° C treatment survival was 80%.

Methyl Farnesoate

Methyl farnesoate was detected in hexane extracts of the hemolymph from female *C. opilio* in all temperature treatments for each sampling period over the 10 month duration of this experiment (Figure 2, A-D). Methyl farnesoate levels in circulating hemolymph at each sampling period in all of the treatments did not significantly change over the egg incubation period. A significant decrease in circulating MF was observed between post extrusion dates and the initial sampling dates in the colder temperatures of -1° C ($p = 0.039$, α 0.05) and 0° C ($p = 0.046$, α 0.05) treatments (Figure 3). There were no significant decreases observed in the 1 or 3° C treatment.

Methyl farnesoate levels (ng/ml) were variable between female *C. opilio*, with some females having "high" MF levels (MF > 40 ng/ml) and some crabs having "low" MF levels (MF < 40 ng/ml). This hormone variation was independent of incubation temperature (Figure 4, A and B). No correlation between crab weight and circulating levels of MF (ng/ml) were observed (Figure 5). Crabs in each temperature treatment, regardless of weight, had either high or low MF levels (ng/ml).

Extrusion

Incubation temperature had a significant effect on the timing of egg extrusion in female *C. opilio*. Within each temperature treatment extrusion began and ended over a period of time which we herein call the extrusion window. The extrusion window characterizes a date range from the onset of extrusion in any one crab of a particular treatment to the day extrusion was complete for all crabs in that treatment. Figure 6 shows that the duration of the extrusion period was significantly shorter in the two warmer temperature treatments (3 and 1° C) when compared to the two colder temperature treatments (0 and -1° C). Egg extrusion in females maintained in the warmer treatments of 3 and 1° C began in late March and early April of 2002. Extrusion began 48 days later in the 0° C treatment and 69 days later in the -1° C treatment. The duration of extrusion increased by 15% in the 1° C treatment relative to the 3° C treatment, 30% in the 0° C treatment relative to the 1° C treatment, and 77% in the -1° C treatment relative to the 1° C treatment (Table 1).

The number of days from first sampling to extrusion increased significantly with decreased temperature ($F_{3,46}$=111.88, $p<0.0001$) with significant differences between the -1° C (256 ± 5.6), 0° C (230 ± 3.1) and 1° C (179 ± 3.6) treatments and between -1, 0 and 3° C (172 ± 2.1) treatments (Figure 7). No significant difference in the number of days to extrusion occurred between the 1 and 3° C treatments.

Ecdysteroids

We validated the ecdysteroid extraction protocol by measuring ecdysteroids in both the methanol and the hexane fractions. The extraction efficiency results for ecdysteroids established that only a small amount (0.17%) of ecdysteroids was extracted from the MeOH with hexane. Ecdysteroid levels at all temperature treatments were not significantly different prior to egg extrusion. Levels of ecdysteroids in three of the four temperature treatments increased after egg extrusion (Figure. 8). Circulating ecdysteroids significantly increased after extrusion in the -1° C ($p = 0.002$, α 0.05), 1° C ($p = 0.017$, α 0.05) and 3° C ($p = 0.014$, α 0.05) treatments (Figure. 9).

Discussion

The shift and contraction in geographic range for *C. opilio* in the EBS from south to north has been linked to changes in water temperature (Orensantz *et al.*, 2004). Snow crabs are physiologically constrained by their thermal limits (Foyle and O'Dor 1989). It has been shown that water temperature affects snow crab population densities (Saint-Marie and Gilbert 1998), influences recruitment of male crabs into the fishery (Zheng and

Kruse 2000) and regulates reproductive processes in female snow crabs (Webb *et al.*, 2006), presumably the fecundity and timing of ovarian maturation in female snow crabs would be influenced.

Our study examined the effect of temperature on the progression of ovarian maturation in *C. opilio* indirectly by evaluating circulating hormones known to regulate reproduction in crustaceans. Increased levels of circulating ecdysteroids are a good predictor of premolt physiology (Chang *et al.*, 1976; Tamone *et al.*, 2005). Similarly, increased levels of the reproductive hormone MF may indicate vitellogenic activity and reproductive function. Circulating MF and ecdysteroid levels were quantified during egg incubation, hatching and extrusion in crabs held at -1, 0, 1, and 3° C. Increases in circulating MF levels in crabs approaching egg extrusion were not observed as previously reported in other studies (Borst *et al.*, 1987; Laufer *et al.*, 1987a; Rodriguez *et al.*, 2002; Reddy *et al.*, 2004; Rider *et al.*, 2006), suggesting that the measurement of this hormone may not be a suitable predictor of ovarian maturation in *C. opilio*. Perhaps during the time we measured MF, vitellogenesis had already been initiated and we missed the predicted increase of MF levels.

Levels of MF decreased significantly after extrusion in crabs incubated at -1 and 0° C. This decrease in circulating MF may be an important chemical cue for hatching, or alternatively, the decrease in MF may correspond to the cessation of vitellogenesis and egg maturation. Circulating levels of MF did not change during the egg incubation period which coincides with vitellogenesis in *C. opilio*. It remains unclear whether MF stimulates vitellogenin synthesis or whether MF stimulates vitellogenin uptake by eggs

during egg maturation or both. In insects, juvenile hormones (structurally similar to MF) aid in the conveyance and/or uptake of vitellogenin into the oocytes (Wilder and Aida, 1995).

Within each temperature treatment, some crabs maintained "high" levels of circulating MF (defined as MF levels > 40 ng/ml) while other crabs maintained "low" circulating MF levels (defined as MF levels < 40 ng/ml) throughout the egg incubation period (Figures 4A and 4B). When crab weight was compared to the average MF levels exhibited by a single crab over the egg incubation time, no correlation within the high or low groups was evident (Figure 5). Age related differences may account for this individual variation in circulating MF levels, as older crabs may have lower levels of circulating MF as they approach senescence. All of the crabs in this study had similar carapace condition (old shelled) which has been loosely related to age, however pseudo cohorts (individuals within a group that differ in age) cannot be distinguished one from another by shell condition. The Bering Sea is considered to have a single population of *C. opilio* (D. Pengilly, ADF& G, personal communication).

Different physiological states may lead to very different hormone profiles in crustaceans. An example of hormones that circulate at different concentrations in crabs of differing physiology has been demonstrated in crabs with a terminal molt (Tamone *et al.*, 2005). Ecdysteroids circulate at low levels in terminally molted snow crabs, which remain in an intermolt stage, and at high levels in juvenile males in an intermolt stage who eventually progress into premolt (Tamone *et al.*, 2005). Methyl farnesoate levels circulate at low levels in unabraided male *L. emarginata* and higher levels in abraided

males (Ahl *et al.*, 1996) suggesting that higher MF levels indicate reproductive capability. It may be that the females with lower MF are at a different life history stage than those with the higher levels of MF, but our data does not reflect these differences, Both groups (high and low MF) extruded eggs after hatching.

The most obvious difference between ovigerous females is whether they are primiparous or multiparous. Primiparous females were excluded from this experiment to decrease the variability and possible confounding results since primiparous females reproduce earlier in the year and could therefore have differing hormone levels when compared to multiparous females. The crabs in this study exhibited ovarian maturation and subsequent extrusion demonstrating that they were all functionally reproductive. It is most unlikely that either group (high versus low MF) was primiparous.

Spermathecal state may also affect female reproductive processes (Elner and Benninger 1992). It is not known if this would cause females to demonstrate variations in circulating MF levels. In this study we confirmed that females with high MF levels and females with low MF levels underwent vitellogenesis as both groups extruded a new clutch after hatching. We do not know if the subsequently extruded clutches were fertilized from stored spermatheca, and we do not know whether MF levels have any indication of sperm availability (ie: whether the lack of males or stored sperm may have had any affect).

While MF levels significantly decreased in crabs after egg extrusion, circulating concentrations of ecdysteroids tended to increase (Figure 8, A-D). Levels of ecdysteroids remained consistently low during the period of egg incubation, but nearing

hatching and extrusion the levels of ecdysteroids become much more variable with a tendency towards increased levels post extrusion. Ecdysteroids significantly increased in all of the temperature treatments with the exception of the 0° C treatment (Fig. 9). It may be of interest that in lower temperature treatments MF decreased as ecdysteroids increased, but not at the 1 or 3°C treatments.

Significant decreases of circulating MF after egg extrusion negatively corresponded to increases of ecdysteroids after egg extrusion, perhaps supporting an antagonistic role for MF on ecdysteroids during reproduction or hatching. MF was found to induce ecdysteroid secretion in an *in vitro* study with cultured y-organs (Tamone and Chang, 1993). Much more remains to be learned about the complexity of the interactions between hormones during reproduction in snow crabs.

Ecdysteroids of ovarian origin are incorporated into maturing eggs and these maternal ecdysteroids may be of physiological significance to the developing crab embryo (Okazaki *et al.*, 1988). Ecdysteroids of ovarian origin are present in developing eggs until the development of the embryo's y-organ is complete. Embryonic sources of ecdysteroids facilitate embryonic development and possibly events related to hatching.

Ecdysteroids circulate at relatively low levels, due to their sequestration in the ovary, during the initial stages of vitellogenesis (Wilder and Aida, 1995). Increased levels of circulating ecdysteroids post extrusion may indicate that the ovaries are no longer taking up maternal ecdysteroids. The interesting implications for a role of ecdysteroids in reproduction should be explored in future studies.

The egg incubation period increased with colder temperature; therefore, the time to extrusion increased significantly ($p < 0.001$, $\alpha = 0.05$) for crabs held at colder temperatures. Prolonged embryonic development would necessarily delay extrusion of the subsequent clutch. In a concomitant study on Bering Sea *C. opilio* reproductive biology , a 17 day increase in length of embryonic maturation was observed per 1° C temperature drop (Webb *et al.*, 2006). Similarly, embryonic maturation rates in *C. opilio* from the Gulf of Saint Lawrence had approximately a 21 day increase per 1° C drop (Moriyasu and Lanteigne, 1998). Extrusion occurs within days of hatching and accordingly we would anticipate synchrony in these coupled events.

Future studies might benefit from efforts to obtain hemolymph samples immediately following both collection and extrusion to establish a baseline for each crab. In addition, more sample times could be added with less volume sampled per time; a larger number of crabs might improve the resolution of the hormone data. With a larger number of females, some could be sacrificed at each time point to document the progress of ovarian maturation in a subset of females within a treatment during the reproductive process.

A promising area for further exploration of female ovarian maturation is to examine circulating vitellogenin (Vg). The development of a vitellogenin assay for snow crabs that would be a quick and sensitive way to ascertain Vg levels in circulation is currently being pursued (S. Tamone, UAS, personal communication). The results from a pilot study with snow crab hemolymph for vitellogenin analysis holds promise. Some samples have been processed with poly acrylamide gel electrophoresis (PAGE) and

stained protein band analyzed by scanning densitometry for the corresponding Vg protein band in circulating hemolymph. Although sample analysis is incomplete, preliminary results have shown an increase in the density of the band corresponding to Vg during the ovarian maturation period. This methodology would be advantageous for its nonlethal approach in detecting Vg from hemolymph samples from the field.

The results of our study support the growing body of information suggesting that reproductive processes in *C. opilio* are affected by temperature. We observed that at lower temperatures the duration of ovarian maturation is significantly increased. Additional research focusing on the regulation of reproduction by temperature is needed to further clarify the reproductive timing for *C. opilio,* as future fluctuations in the NBT of the EBS may continue to affect recruitment.

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Treatment	Extrusion period	Mean extrusion date Duration (days) ± SEM	Duration (days) % increase in duration	Avg. Range MF (ng/ml) Min ± SEM Max ± SEM	% Survival
-1° C (n=15)	6/9/02 - 8/17/02	6/29/02 69 (± 4.1)	69 77 %	0.40 (± 23.9) 163.31 (± 25.3) (n = 7)	70
0° C (n=13)	5/18/02 - 6/27/02	6/9/02 39 (± 3.1)	39 50 %	0.14 (± 21.0) 133.62 (± 24.4) (n = 6)	90
1° C (n=11)	4/4/02 - 5/4/02	4/19/02 30 (± 3.6)	30 15 %	1.80 (± 1.6) 97.50 (± 23.6) (n = 4)	70
3° C (n=16)	3/30/02 - 4/25/02	4/12/02 26 (± 1.7)	26 NA	0.39 (± 14.9) 135.53 (± 20.9) (n = 7)	80

Table 1: Summary of data according to treatment. No. of crabs, extrusion period dates, mean extrusion date (± SEM), increase in duration (days) of extrusion of a treatment in relation to the next warmest treatment, average range (± SEM) of MF (ng/ml), percent survival.

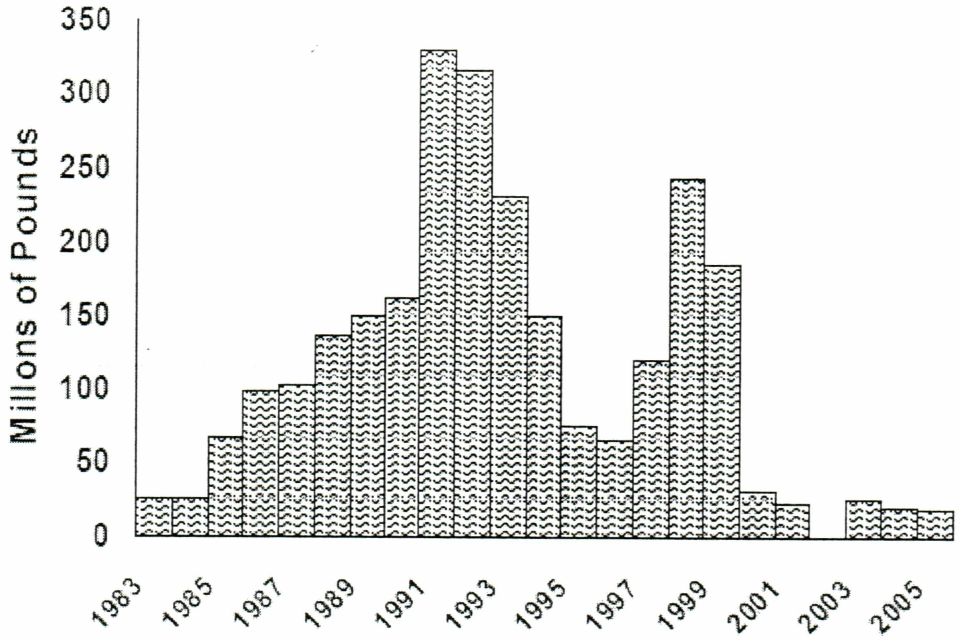

Figure 1. *Chionoecetes opilio* Eastern Bering Sea harvest 1983-2005. Bars represent Alaska fish and Game data for harvest in millions of pounds.

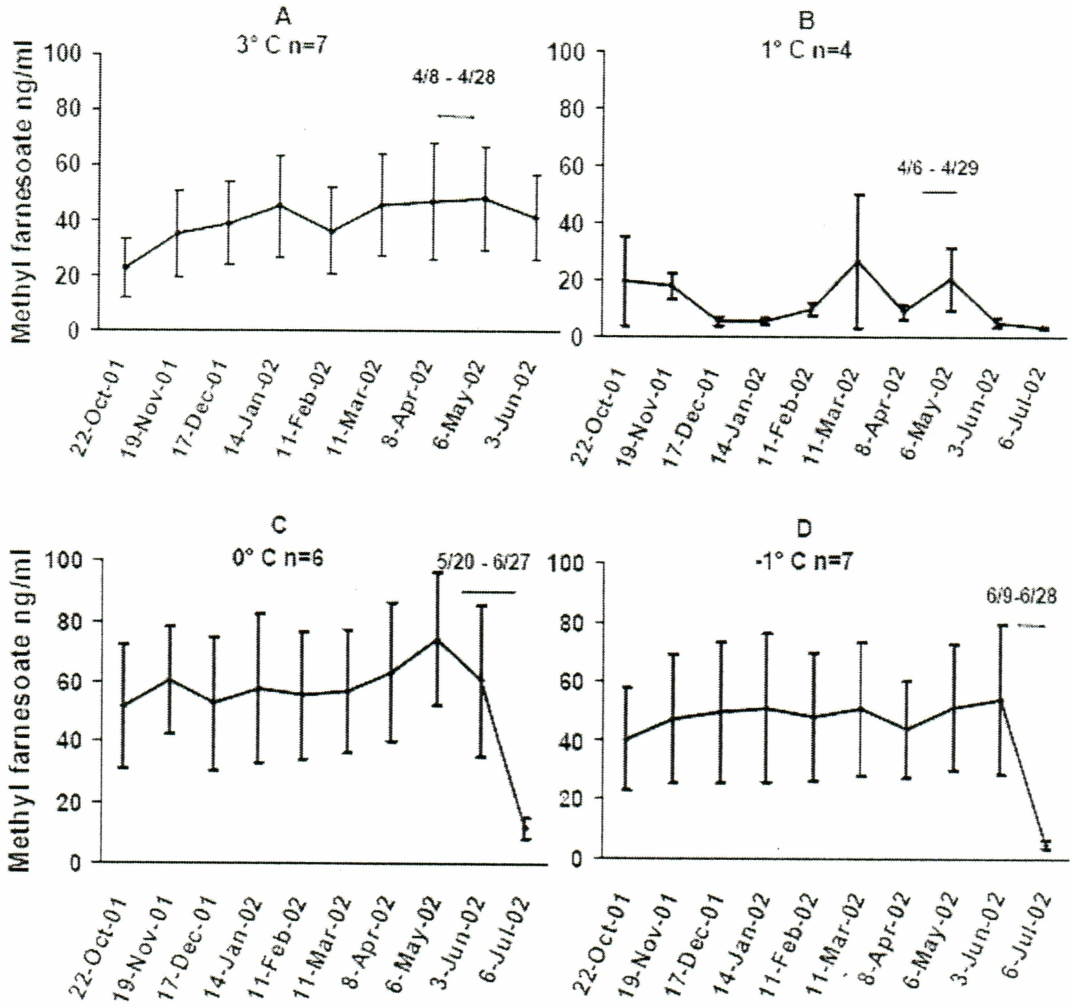

Figure 2. Average circulating methyl farnesoate levels by treatment. A. 3° C (n=7), B. 1° C (n=4), C. 0° C (n=6), D. -1° C (n=7). Error bars represent ± SEM. Arrows represent date range of extrusion for all crabs within that treatment.

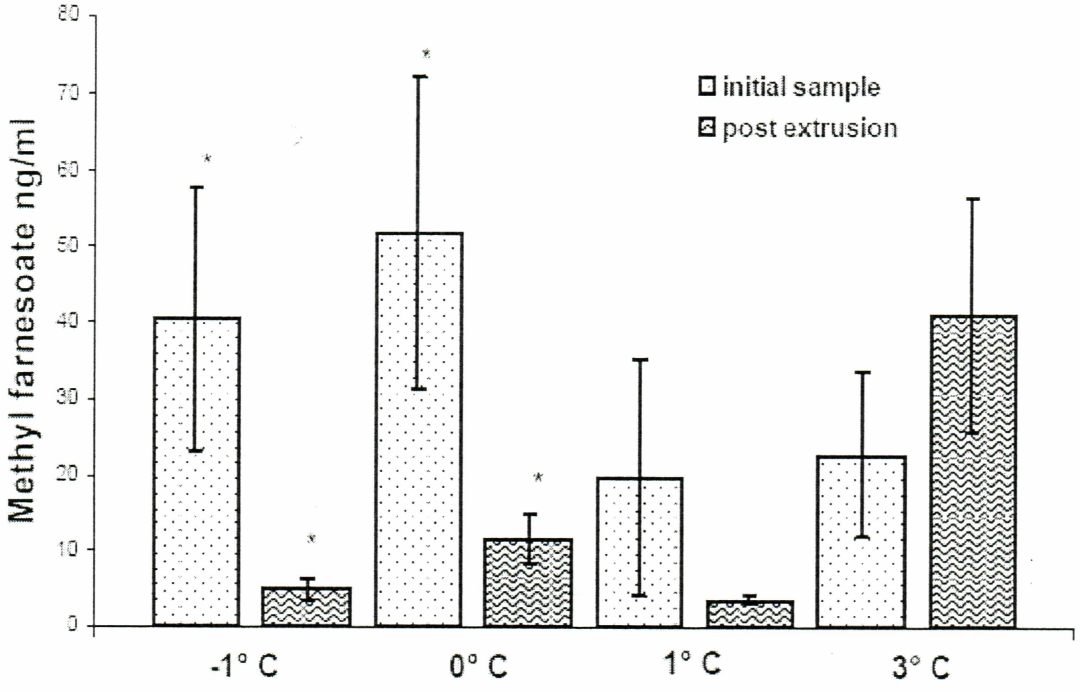

Figure 3. Circulating levels of methyl farnesoate prior to and after egg extrusion. Values represent the mean ± SEM. Significance (*) was found within treatments by paired t-Test in the -1° C ($p=0.039$, $\alpha=0.05$) and 0° C ($p=0.046$, $\alpha=0.05$).

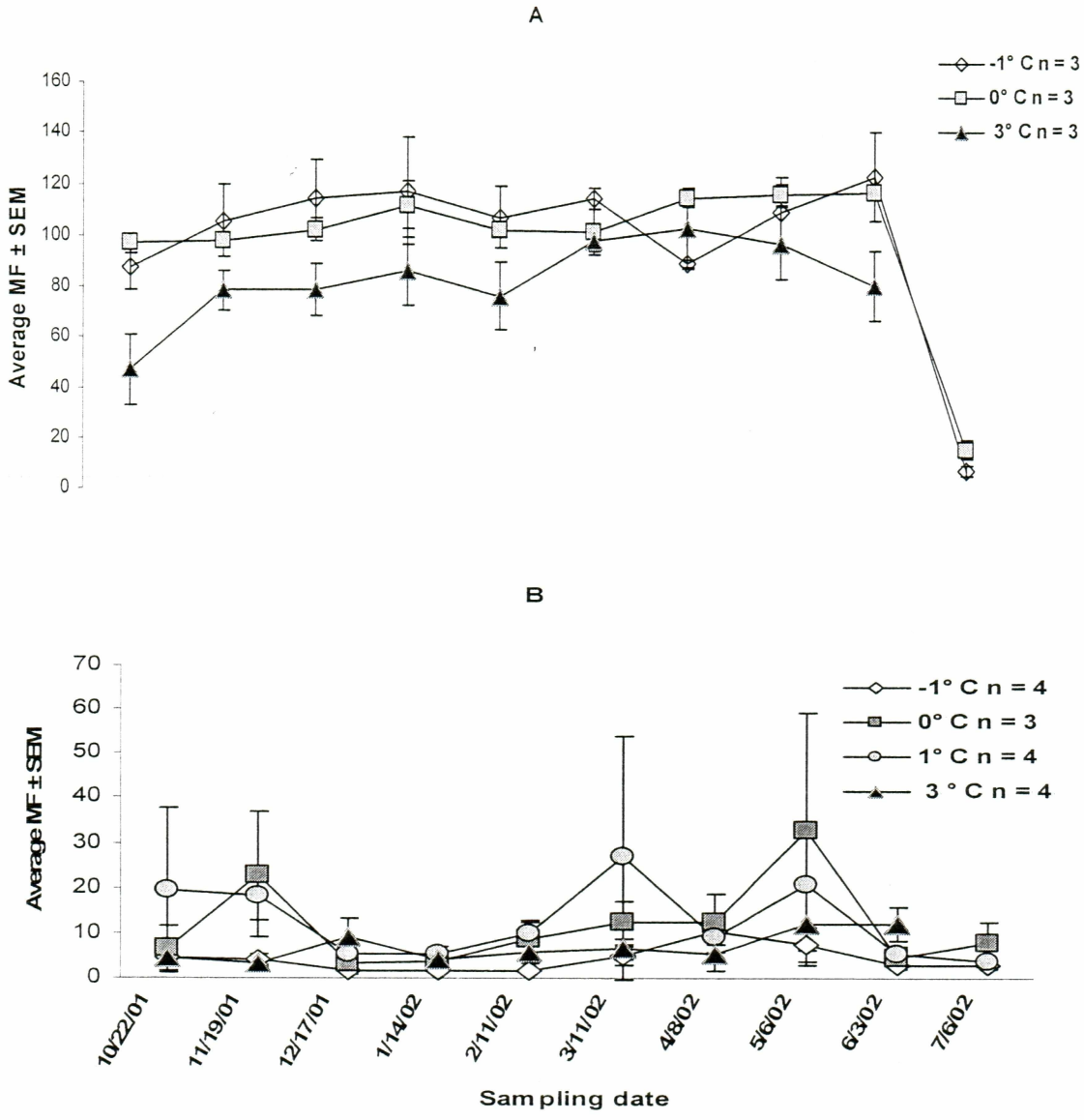

Figure 4. Circulating methyl farnesoate levels in female snow crab. "A" represents a subset of crabs with average concentrations of MF > 40 ng/ml ± SEM. "B" represents a subset of crabs with lower average concentrations of MF < 40 ng/ml ± SEM.

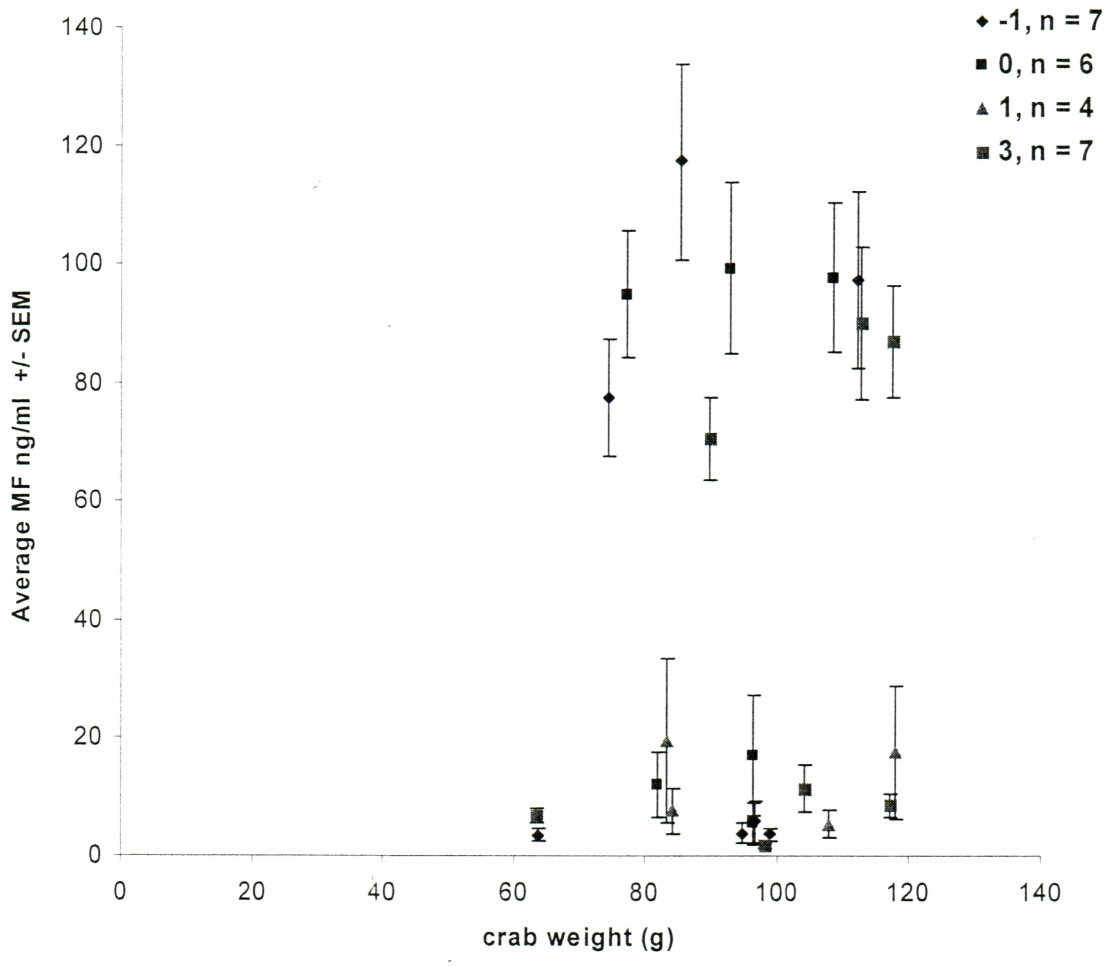

Figure 5. Methyl farnesoate levels in individual snow crabs versus crab weight. Values represent the average MF concentration (± SEM), of individual female crabs over the duration of the experiment.

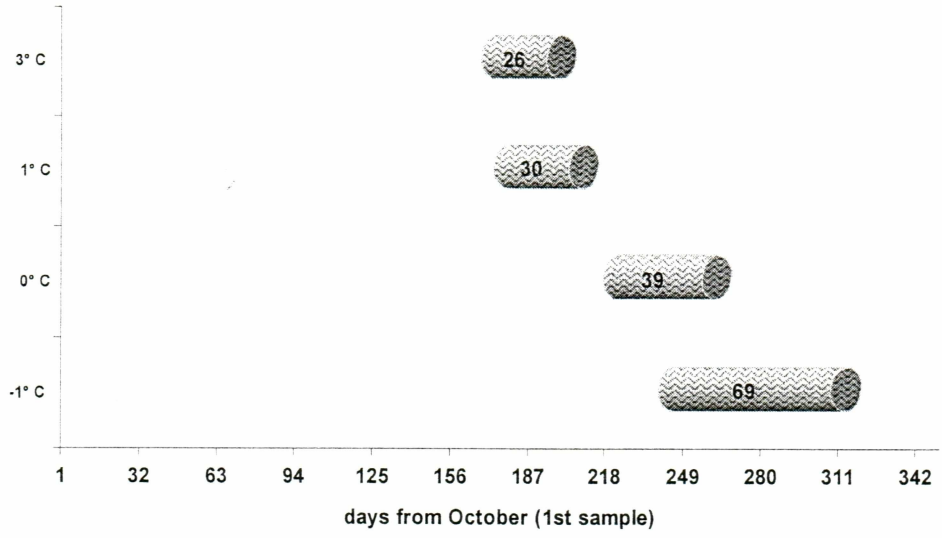

Figure 6. Onset and duration of egg extrusion. Bars represent the extrusion window in days for the four temperature treatments: -1° C (n = 15), 0° C (n = 13), 1° C (n = 11) and 3° C (n = 16). Increases in duration of extrusion occurred as temperatures decreased.

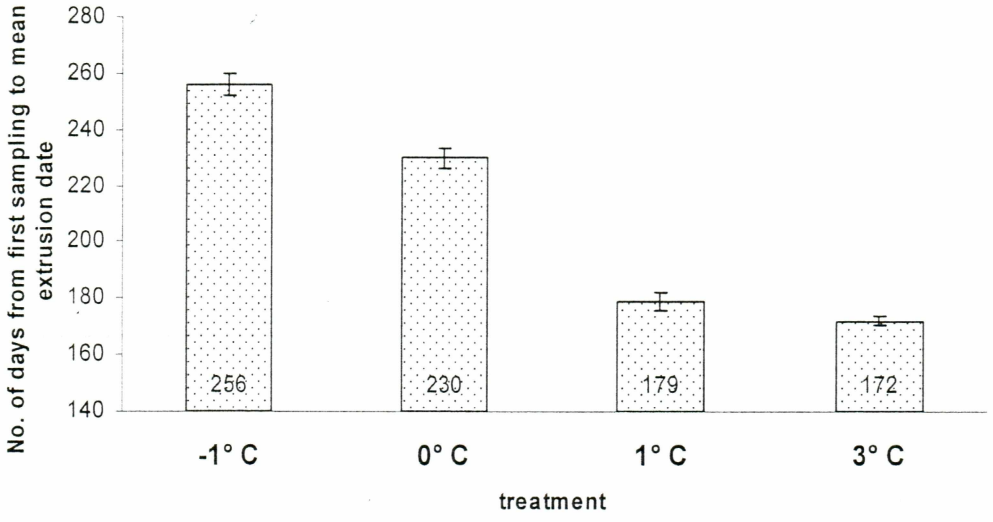

Figure 7. Average extrusion duration by treatment. Bars represent the average number of days ± SEM from initial hemolymph sample (October) to the time of egg extrusion, for each temperature treatment -1° C (n = 15), 0° C (n = 13), 1° C (n = 11), 3° C (n = 16). Different letters represent significance at $\alpha = 0.05$.

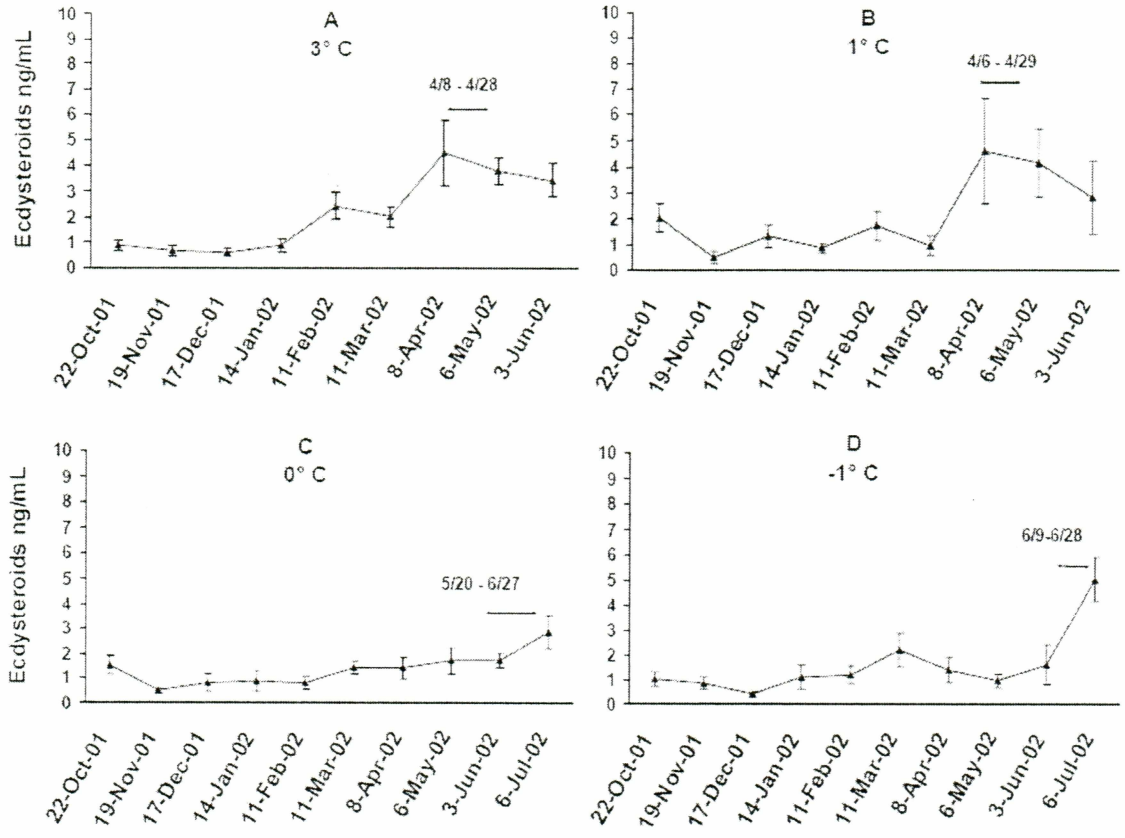

Figure 8. Circulating ecdysteroid levels in snow crabs at four different temperatures. A. 3° C (n = 8), B. 1° C (n = 7), C. 0° C (n = 9), D. -1° C (n = 7). Values represent the mean ± SEM.

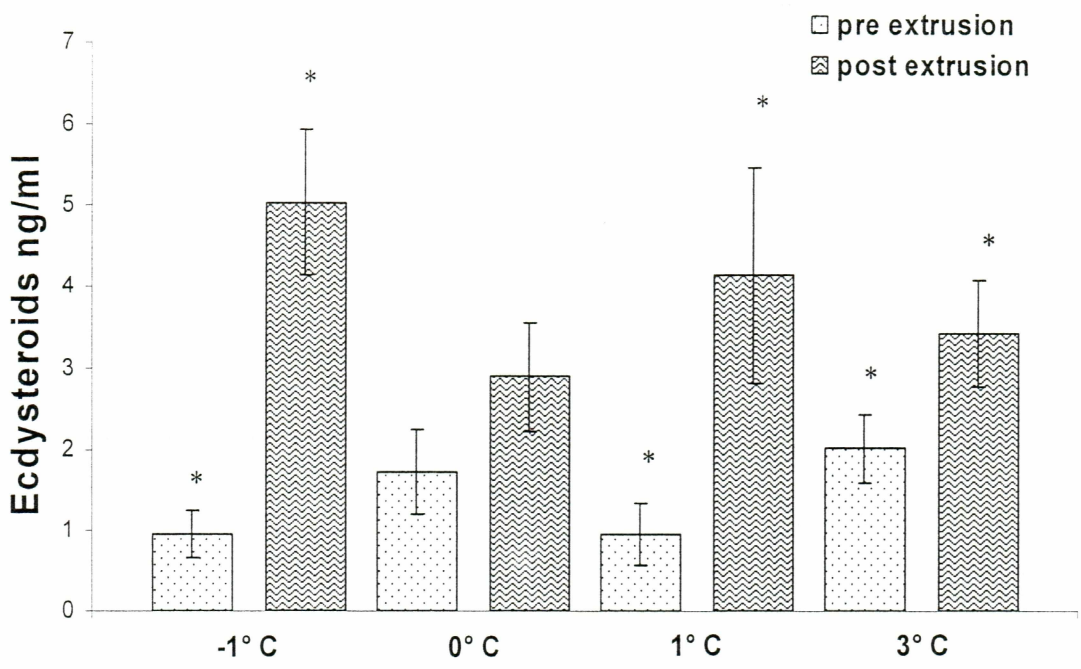

Figure 9. Circulating ecdysteroid levels in snow crabs prior to and after egg extrusion. Bars represent mean circulating ecdysteroid levels (ng/ml ± sem) in female crabs before and after egg extrusion. Comparison within treatments indicates significance (*) at -1° C ($p = 0.002$, at α 0.05) and 1°C ($p = 0.017$, at α 0.05) and 3 °C ($p = 0.014$, α=0.05) treatments.

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Conclusion

The impacts water temperature may have on reproduction, fecundity, and recruitment for the snow crabs in the Eastern Bering Sea population is a vital question for this fishery, as near bottom temperatures fluctuate. A concurrent research project, using the same individual snow crabs as this study, reports that zoeae morphology is affected by variations in incubation temperature (Webb *et al.*, 2006).

This study was designed to characterize aspects of the reproductive cycle of female snow crabs and determine what, if any, temperature effects may have on hormonal processes. We successfully extracted and quantified ecdysteroids and for the first time in snow crabs, methyl farnesoate, and we characterized the levels of these hormones through a full reproductive cycle. Although methyl farnesoate levels showed no pattern between temperature treatments we found longer ovarian maturation periods at colder temperatures. Future studies that provide a comparison of ovarian development, spermathecal load, methyl farnesoate and vitellogenin levels from females within a single experiment may illuminate, among other things, the timing and perhaps function of methyl farnesoate during the reproductive process. Characterization of vitellogenin levels is a compelling research direction for understanding snow crab reproduction.

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